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ADDING GELLING AGENTS TO COTTON OVULE CULTURE MEDIA LEADS TO SUBTLE CHANGES IN FIBER DEVELOPMENT

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SUMMARY

Young cotton (*Gossypium hirsutum*) ovules will produce fiber in vitro when floated on a defined culture medium. Our laboratory is interested in examining the effects of altered gravity environments on fiber development as a model for the effects of gravity on cell expansion and cellulose biosynthesis. Since liquid culture media are unsuitable for altered gravity experiments, addition of gelling agents to cotton ovule culture media is necessary. In this study we have systematically examined the effects of four gelling agents at several concentrations on fiber production in culture. A rapid screening method using toluidine blue O staining indicated that after 3 wk in culture, fiber growth on 0.15% (wt/vol) Phytagel™ medium was similar to fiber growth on liquid medium. More detailed analysis of fiber development revealed that fiber length was not influenced by the addition of Phytagel™. Accumulation of cellulose, however, was reduced 50–60% compared with fibers produced in liquid media after 3 wk in culture. The fiber cellulose content rose with additional time in culture for both solid and liquid media treatments. By 4 wk in culture, the difference in cellulose content of fiber cell walls grown on solid versus liquid media was less than 20%. This variance in growth response on gelled media could be due to differences in media matric potential, to the immobility of ions trapped within the gel, or to toxicity of contaminants copurifying with Phytagel™. By identifying why ovule growth and fiber cellulose biosynthesis are reduced in cultures grown on gelled media, it will be possible to reveal new information about these processes in a system that is less complicated than physiological systems at the whole plant level.

Key words: cellulose synthesis; fiber growth; *Gossypium*; gellan gum; Phytagel™.

INTRODUCTION

Many of the nutritional and hormonal requirements for cotton fiber development were initially determined by in vitro culture of ovules on a defined liquid medium (Beasley and Ting, 1974). Typically, ovules are harvested from young ovaries, 2 d postanthesis (DPA) or younger, and floated on top of the culture medium for 2 to 4 wk. Fibers are single-celled trichomes that differentiate from the ovule's epidermal surface. Within a few d in culture, fiber growth is evident by visual inspection. Fiber cells continue to elongate for 10–12 d and, at maturity, are characterized by a high cellulose content in the cell wall.

Our laboratory has used the cotton ovule culture system on many occasions to address fundamental questions about cotton fiber growth and development (Mellon and Triplett, 1989; Triplett et al., 1989; Triplett and Timpa, 1995; Andersland et al., 1998; Triplett, 1998). Of special interest to our group is determining how the organization of cellulose microfibrils is regulated by the cell. This question has significant economic impact for the textile industry since the organization of cellulose microfibrils in cotton fiber cell walls is one of the most important determinants of cotton fiber strength. Cell wall polymer organization in cotton fiber is influenced by a network of microtubules and microfilaments in the cytoplasm known as the cytoskeleton (Seagull, 1986). Perturbation of cytoskeletal structure by adding cytoskeletal inhibitors (taxol, cytochalasin B and D, colchicine, oryzalin, trifluralin) to ovule cultures leads to alterations in cellulose microfibril organization (Seagull, 1990).

In reviewing the literature to identify other mechanisms for modifying cytoskeletal structure without the use of inhibitors, we discovered that exposure to microgravity or hypergravity often changes cytoskeletal structure in eukaryotic organisms (Thomason et al., 1992; Wilfinger and Hymer, 1992; Hilaire et al., 1995; Clejan et al., 1996; Volkmann and Tewinkel, 1996). Microgravity is the condition that orbiting space craft undergo during their free-fall orbits around earth. Hypergravity is any condition in which the effective gravitational force is greater than 1 $\times$g. There have been many attempts to grow plants in microgravity during the last two decades of the U.S. space program. In summary, the results from a number of studies suggest that plant cell wall composition and structure is altered with microgravity exposure (Dutcher et al., 1994; Kordyum, 1997).

Culture of cotton ovule cultures in microgravity seemed like a fruitful approach for monitoring the effects of microgravity and hy-

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[2]Names of companies or commercial products are given solely for the purpose of providing specific information; their mention does not imply recommendation or endorsement by the U.S. Department of Agriculture over others not mentioned.

pergravity conditions on plant cell wall development. Additionally, we could obtain new information about the relationship between cytoskeletal structure and cell wall organization. Before such studies could begin, however, the ovule culture conditions had to be modified. As originally described by Beasley and Ting (1974), cotton ovule cultures are initiated by floating immature ovules on liquid culture medium. In microgravity, these culture conditions would not be suitable, so we initiated this study to determine if adding a gelling agent to culture media would change the capacity of the ovules to produce fiber or alter the physical properties of the fiber.

Numerous examples have been documented about how various gelling agents or gel concentrations lead to differences in plant differentiation in vitro (Williams, 1993; Hadeler et al., 1995; Smith and Spomer, 1995; Williams, 1995). Rarely have investigators discovered the underlying physiological basis for the observed differences. In light of the possibility that creating a solid medium for ovule culture growth might alter fiber development, we began characterizing the in vitro conditions that will generate fiber of good quality and quantity and still be suitable for hypergravity or microgravity conditions.

Materials and Methods

Plant material. *Gossypium hirsutum* (DPL 90ne) was grown in New Orleans, LA in a greenhouse. Stages of development are expressed relative to the day of anthesis (DOA) as the number of days postanthesis (DPA). Cultures were initiated from ovules harvested on the morning of anthesis.

Chemicals and reagents. All chemicals were reagent grade unless otherwise noted. The following gelling agents were used to solidify media: Bacto-agar[2] (Difco Laboratories, Detroit, MI), agarose (electrophoresis grade, GIBCO BRL, Grand Island, NY), Phytagel™ and Agargel™ (Sigma Chemical Co., St. Louis, MO).

Ovule culture. Cultures were initiated as previously described (Triplett, 1998) with the following modifications. Cultures were grown at 32° C on liquid Beasley–Ting media (BT) (Beasley and Ting, 1974) or on BT media containing one of the gelling agents. A single layer of Micropore™ surgical tape (3M Healthcare, St. Paul, MN) was wrapped around each box. One boll (approximately 30 ovules) was used for each dish with four boxes per treatment.

Media hardness testing. Petri dishes containing 50 ml BT medium with several concentrations of gelling agents were prepared in triplicate. Media were allowed to cool to room temperature and were held 16 h in a laminar flow hood. Hardness testing was conducted with a TA-XT2 Food Texture Analyzer (StableMicro Systems, Surrey, United Kingdom) with a 5-mm-diameter flat probe. Three measurements were made for each concentration of agent, with a fresh petri dish for each independent measurement. Measurements were made in the center of each dish to avoid edge effects. The probe penetration speed was 1 mm s^{-1}. The penetration force was measured in units of grams force with higher values indicative of greater strength gels.

TBO staining. A semi-quantitative method for measuring fiber development with the stain toluidine blue O (TBO)(Beasley et al., 1981) was used to assess the level of fiber production by ovule cultures grown under various conditions. Fifty ovules from each treatment were stained with 0.02% (wt/vol) TBO simultaneously in a disposable plastic specimen cup. After being washed with water to remove unbound TBO, individual ovules were placed in test tubes and destained for 1 h in 50% ethanol with gentle shaking. Replicate aliquots of the destaining solution from each tested ovule were placed into wells of a 96-well microtiter plate. The absorbance at 650 nm was read by a ThermoMax (Molecular Devices Corp., Menlo Park, CA) microtiter plate reader with duplicate, twofold serial dilutions of 0.01% TBO as standards. The A_{650nm} of 100 µl of 0.01% (wt/vol) TBO was arbitrarily defined as 100 TBO units in this study. Comparisons between mean values for each treatment and distributions of individual values were made. Distributions of TBO values were determined by pooling individual ovule TBO unit values into classes. The percentage of individuals in each class were plotted as a function of class number for each treatment. For this study the class numbers were defined as follows: Class 1, <1 TBO unit; Class 2, 1–5 TBO units; Class 3, 6–10 TBO units; Class 4, 11–15 TBO units; Class 5, 16–20 TBO units; Class 6, 21–25 TBO units; Class 7, 26–30 TBO units; Class 8, 31–35 TBO units; Class 9, 36–40 TBO units; Class 10, 41–45 TBO units.

TABLE 1

MEDIA HARDNESS AND FIBER GROWTH FOR SEVERAL CULTURE CONDITIONS CONTAINING DIFFERENT CONCENTRATIONS OF VARIOUS GELLING AGENTS

Gelling agent	Hardness[a] (grams force)	Fiber growth[b] (TBO units)
0.1% Phytagel™	215.5 ± 32.5	14.46 ± 8.4
0.15% Phytagel™	582.9 ± 49.4	12.40 ± 6.8
0.2% Phytagel™	994.3 ± 38.2	3.46 ± 5.0
0.25% Agargel™	214.0 ± 77.6	7.91 ± 5.7
0.35% Agargel™	798.2 ± 51.5	5.23 ± 1.27
0.45% Agargel™	1385.4 ± 37.5	1.17 ± 4.4
0.5% agar	444.8 ± 34.4	13.32 ± 8.0
0.5% agarose	2974.6 ± 36.4	9.12 ± 6.9
No gelling agent	N/A	13.51 ± 3.9

[a]Hardness was measured with a Food Texture Analyzer.

[b]Fiber growth was measured by the toluidine staining method (Beasley et al., 1981) and is reported as the means ± standard deviation of 50 ovules per treatment.

Hypergravity simulation. Phytagel™-hardened media at two different concentrations [0.1% (wt/vol) and 0.15% (wt/vol)] was prepared in Magenta boxes (Magenta Corporation, Chicago, IL). There were four replicate cultures initiated for each concentration of gelling agent. Thirty DOA ovules were placed on the solid media surface. Cultures were held for 7 d in the swinging bucket rotor (TH-4) of a Beckman model TJ 6-R low-speed centrifuge (Beckman Instruments, Palo Alto, CA) placed inside a 30° C environmental chamber. The speed of the centrifuge was adjusted so that relative centrifugal force at the surface of the solid BT media was 2 ×g.

Ovule and fiber measurements. Procedures for measuring the number of ovules producing fiber, the amount of fiber per ovule, fresh and dry weights of fiber and ovules, fiber length, and fiber cellulose content were conducted as previously described (Triplett, 1998).

Results and Discussion

Four compounds capable of solidifying cotton ovule culture media were selected for comparison in this study: agar, agarose, Phytagel™, and Agargel™. Agar is a complex polysaccharide mixture isolated from the algal family Rhodophyceae. Agarose is purified from agar and consists of 1,3-linked β-D-galactopyranose and 1,4-linked 3,6-anhydro-α-L-galactopyranose. Phytagel™, a commercial preparation often used in plant tissue culture applications, is composed of gellan gum, a product of bacterial fermentation. Agargel™ is a mixture of agar and Phytagel™.

When gelling agents were added to cotton ovule culture media, the resulting hardness of the medium was dependent upon the identity and concentration of gelling agent used (Table 1). The Texture Analyzer measures the compressive deformation of the gel at the point that the probe punctures the gel surface (Kampp, 1995). By measuring the hardness of different media preparations, it was possible to adjust the concentration of a gelling agent to obtain a hardness value similar to the value obtained for another gelling agent. For example, the hardness of 0.1% Phytagel™ was similar to the hardness of 0.25% Agargel™.

Similarly, the amount of fiber produced by the ovule cultures as determined by TBO staining was related to both the identity and concentration of agent used to solidify the medium (Table 1). Lower concentrations of gelling agents had better fiber growth as measured by mean TBO staining values. Of the gelling agents tested in this study, Phytagel™ performed optimally. Under conditions where the

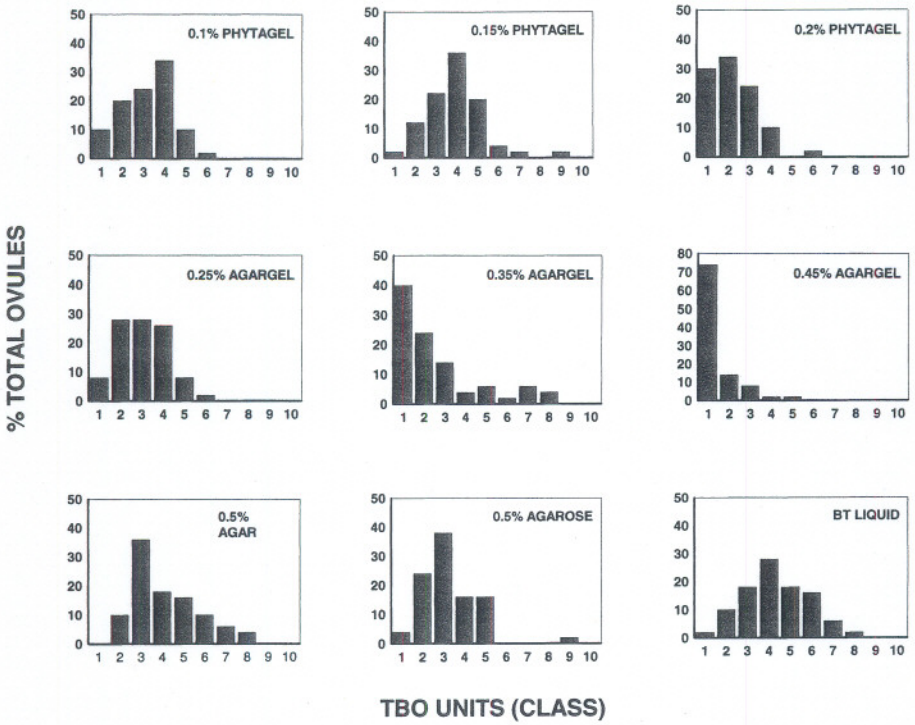

FIG. 1. Fiber growth on media containing different gelling agents compared with liquid Beasley–Ting medium. Fiber growth on individual ovules was measured by toluidine blue O (TBO) staining. Dye uptake was measured for each ovule. Values for TBO uptake were grouped into 10 classes based upon uptake intensity.

medium hardness was similar, the amount of fiber on Agargel™ plates was approximately half the amount grown on Phytagel™ media as measured by TBO staining. Ovules cultured on plates containing agar or agarose developed more callus than liquid BT medium control cultures where no callus formed. This observation is consistent with the earliest reports on cotton ovule cultures in which the addition of agar resulted in increased callus growth (Beasley and Ting, 1973). No callus tissue grew in ovule cultures grown on Phytagel™ or Agargel™.

Not only were average TBO values affected by gelling agent and concentration, the distribution of individual values also indicated a dependence on culture medium components (Fig. 1). The distribution of TBO stain values for liquid BT medium was a Gaussian distribution. Addition of gelling agents to media at higher concentrations led to a shift in the distributions to lower classes. Phytagel™ at 0.1% and 0.15% had TBO class distributions that were most similar to the distribution for liquid BT medium. From this preliminary screen with TBO staining, all gelling agents were omitted from further consideration except 0.1% and 0.15% Phytagel™.

Future plans for using gelled media include examining the effects of hypergravity on fiber development in ovule culture. Therefore, a preliminary experiment was conducted to determine which concentration of Phytagel™ would support the weight of day-of-anthesis cotton ovules under hypergravity ($2 \times g$) conditions. In three of the four replications, all ovules pelleted into or through the solid media when Phytagel™ was used at 0.1% (wt/vol). In contrast, all of the ovules remained on the surface of the solid media when Phytagel™ was used at 0.15% (wt/vol). For all subsequent experiments, comparisons were made of ovule cultures grown on liquid BT medium with cultures grown on BT medium containing 0.15% (wt/vol) Phytagel™ (Fig. 2).

Liquid media and media solidified with 0.15% Phytagel™ were equally effective in initiating high densities of fiber growth on in vitro cultured ovules (Table 2). Cotton ovules grown on liquid BT medium attained higher fresh weight and dry weight values than ovules cultured on BT medium containing 0.15% (wt/vol) Phytagel™. Ovules grown on BT medium solidified with 0.15% (wt/vol) Phytagel™ attained only 48% of the dry weight of ovules grown in liquid. The percent water content of ovules grown on both media was similar. Fiber length was identical for ovules grown in liquid and those grown on solid media. Fiber length measurements, however, were taken for the longest group of fibers growing on an ovule and may not adequately reflect differences in the distribution of fiber lengths on an ovule. Fiber fresh weight per ovule was reduced by 53% when ovules were grown on solid media. Similarly, fiber cellulose content was reduced by 48% in cultures grown on Phytagel™-gelled media. In summary, while the degree of fiber elongation under the two growth conditions was quite similar, accumulation of ovule mass, fiber mass, and cellulose content was reduced in ovules grown on BT medium with Phytagel™ compared with liquid culture.

The similar reductions in ovule and fiber mass and fiber cellulose content suggest that addition of Phytagel™ to BT culture medium suppressed some fundamental aspect of ovule growth. Numerous investigators have found that the addition of a gelling agent to plant culture media can influence plant differentiation in vitro (reviewed by Smith and Spomer, 1995; Williams, 1995). When a gelling agent is added to culture media, all of the media components, including water, become enmeshed within the complex matrix of the gel. Diffusion and transport through the gel may be altered in gels compared with liquid media. In the past, it was assumed that the differences in plant growth on different concentrations of gelled substrates was due to differences in water availability to plant tissues. A recent

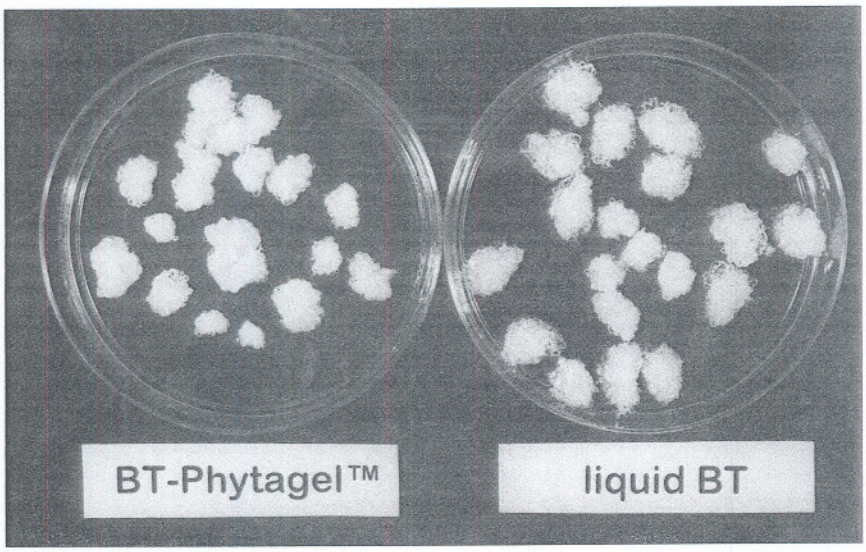

FIG. 2. Ovule cultures grown on liquid Beasley–Ting medium (*A*) or on Beasley–Ting medium containing 0.15% (wt/vol) Phytagel™ (*B*) after 21 d in culture.

study indicates that water potential and conductivity are affected negligibly by gelling agents over the range of concentrations typical for plant cell culture (Spomer and Smith, 1996). As a result, it is unlikely that the different distributions of TBO classes for different gelling agents are due solely to water availability differences, unless ovules are extremely sensitive to minute changes in water potential differences. Conversely, the difference in water potential due primarily to matric potential differences between solid and liquid media must be contributing to the observed differences in ovule culture growth. Another possibility is that there are other factors changing coordinately with gelling agent concentration that lead to the reported differences in developmental response in vitro.

In most cases, gelling agents also contribute small amounts of inorganic ions to plant culture media. The fermentation medium for producing gellan gum contains D-glucose, potassium phosphate, ammonium nitrate, soy protein hydrolyzate, magnesium, iron, copper, zinc, molybdenum cobalt, manganese, and boron (Kang and Pettitt, 1993). When gellan gum is precipitated from fermentation tanks with alcohol, bacterial culture medium becomes trapped in the product, leading to contamination of the gelling agent. In the case of Phytagel™, the ions representing the highest percentage of contamination are listed in Table 3. Also listed in Table 3 are the concentrations of these elements in BT medium before and after the addition of

TABLE 2

GROWTH PROPERTIES OF COTTON OVULES AND FIBERS GROWN IN VITRO ON LIQUID BT MEDIUM OR ON BT MEDIUM + 0.15% PHYTAGEL™

Property	Liquid BT	BT + 0.15% Phytagel™
Ovules with fiber (%)	100	100
Ovules fresh weight (mg/ovule)	139.0 ± 15.74	79.7 ± 10.97
Ovule dry weight (mg/ovule)	9.4 ± 2.1	4.48 ± 1.17
Ovule water content (%)	93.2	94.4
Fiber length (cm)	1.72 ± 0.46	1.72 ± 0.38
Fiber weight/ovule (mg/ovule)	6.76 ± 1.64	3.61 ± 0.65
Cellulose content (% dry wt)	29.30 ± 7.89	14.10 ± 5.07

Values are reported as the mean ± standard deviation of 40 ovules per treatment.

Phytagel™ (values reported are from the specific lot of Phytagel™ used in this study) and the increase in each element as a result of Phytagel™ addition to a final concentration of 0.15% (wt/vol). The concentration of two divalent cations, Ca^{+2} and Mg^{+2}, increased 1.08 and 1.09-fold, respectively, as a result of Phytagel™ addition. Similar low increases in potassium, phosphorus, and sulfur concentration

TABLE 3

INCREASE IN CONCENTRATION OF SELECTED INORGANIC COMPONENTS OF BT MEDIUM AS A RESULT OF PHYTAGEL™ ADDITION

Element	Concentration in Phytagel™[a] (%)	Concentration in BT medium (m*M*)	Concentration in BT + Phytagel™[b] (m*M*)	Fold increase in element concentration
K	1.72	52.01	52.67	1.01
Ca	0.62	3.00	3.23	1.08
Na	0.69	0.062	0.512	8.26
Mg	0.23	2.00	2.18	1.09
P	0.10	2.00	2.05	1.02
S	0.04	2.16	2.18	1.01

[a]Inductively coupled plasma emission spectrometry analysis of Lot #13H0072 in Sigma Chemical Company technical records.
[b]This calculation is based on a final concentration of Phytagel™ at 0.15% (wt/vol).

also resulted from Phytagel™ use. In contrast, sodium ion concentration increased to 0.512 m*M*, a value 8.26 times higher than the concentration in liquid BT medium. Even though sodium ion concentration increased, it is unlikely that the diminished growth of the ovule and reduced cellulose production by the fiber is explained totally by an increase in sodium ion concentration. Cotton is a salt-tolerant plant and in vitro-grown tissues can be exposed to 50–100 times higher levels of sodium ion before significant stress effects are observed (Gossett et al., 1994). Recently, a method was developed for removing cations from Phytagel™ by a rapid two-step method (Doner, 1997). We plan to culture cotton ovules on medium gelled with Phytagel™ purified by this method to determine directly if the observed differences in growth properties were the result of a higher sodium ion concentration.

Gellan gum requires divalent cations, especially Ca^{+2} and Mg^{+2}, for gel formation to occur (Kang and Pettitt, 1993). In addition, gel strength is highly dependent on divalent cation concentration in the medium. To test if sufficient levels of divalent cations were present in the lot of Phytagel™ used for this study, we added 0.15% Phytagel™ to distilled water, adjusted the pH to 6.0, and autoclaved it in the same fashion that media had been prepared. Under these low ionic strength conditions the Phytagel™ stayed in a liquid phase after cooling. This observation suggests that the concentration of Mg^{+2} and Ca^{+2} contaminants in this lot of Phytagel™ were not sufficient to permit full gel formation. As a result, the divalent cations added in the BT medium participated in gel formation and may not have been fully available for ovule nutrition. During the 3-wk culture period, it is possible that divalent cations were either participating in the gel structure and were thus immobile or were removed from the area proximal to the tissue and could not adequately move toward the growing tissue. Depletion of resources near the ovule during later stages of culture seems a more likely explanation since maximum fiber cell elongation was not affected by the addition of Phytagel™ to the culture medium (Table 2). We will test this possibility in the future by moving ovule cultures to fresh media in the middle of the 3-wk culture period. Additionally, cellulose biosynthesis is also dependent on Mg^{+2} concentration (Delmer and Amor, 1995), so that suboptimal concentrations of this cation could negatively impact cellulose production.

It is possible also that the presence of a gelling agent may influence the medium pH differently than a liquid medium (Williams, 1993). Media containing Phytagel™ were prepared from the identical batch of BT medium used as the liquid medium control and were adjusted to pH 6.0. After autoclaving there were no differences in the pH between liquid BT and BT-Phytagel™; however, the pH of both media dropped to 5.3. These more acidic conditions can immobilize certain ions in the gel matrix, making them less available to the plant tissue (Williams, 1993).

With additional time in culture, ovules growing on BT-Phytagel™ were able to produce fiber with a higher cellulose content (Fig. 3). By 4 wk in culture, 30% of the fiber cell wall weight was due to cellulose in BT-Phytagel™-grown fiber compared with 37% for liquid BT-grown cultures. At 4 wk, the difference in fiber cellulose content in solid versus liquid-grown cultures was less than 20%. This 20% difference in cellulose content persisted even after 5 or 6 wk in culture. Nevertheless, we propose that by extending the time period for culture from 3 to 4 wk, cotton ovule cultures should produce enough cellulose under 1 ×g conditions to permit their use under altered gravity environments in experiments to evaluate gravity effects on cell wall biogenesis.

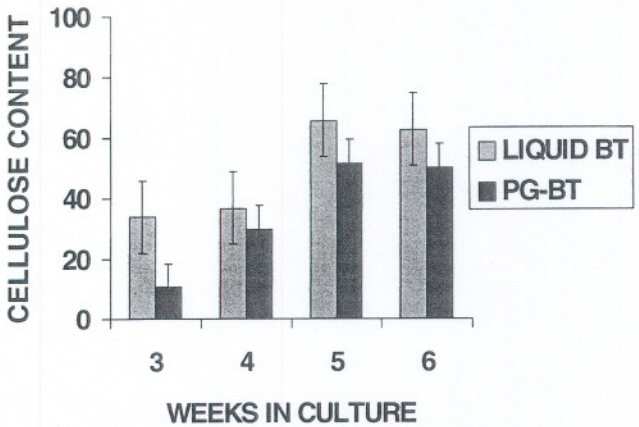

FIG. 3. Cellulose content of fiber grown in ovule culture on liquid Beasley–Ting medium or 0.15% Phytagel™–Beasley–Ting medium after 3 to 6 wk in culture. Cellulose content is expressed as the percentage of the fiber dry weight that is cellulose.

The observation that cellulose content is reduced by one-half under conditions in which the physical phase of the culture media is solid rather than liquid suggests that this in vitro culture system could be used to identify agronomically important traits in cotton. A reduction in fiber cell wall thickness from decreased production of cellulose results in fibers that cannot be spun into yarn easily, that do not dye uniformly, and that create imperfections in fabrics. As demonstrated here, the physiological basis for reduced cellulose production by the fiber cells can be studied in vitro simply by changing the physical phase of the culture medium. Differential display analysis (Liang et al., 1994; Kim and Triplett, unpublished) could be used to identify quantitative differences in gene expression under the two culture conditions. Such approaches may lead to the characterization of specific genes that are involved in producing superior quality cotton fiber.

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